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(54) Title of the Invention: Process for measuring inorganic phosphorus

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DESCRIPTION

1. TITLE OF THE INVENTION Process for measuring inorganic phosphorus

2. Claims

(1) A process for measuring inorganic phosphorus characterized in: allowing a reaction between sucrose and inorganic phosphorus in the presence of sucrose phosphorylase; producing NAD(P)H from NAD(P) through combining the produced glucose-1-phosphate with phosphoglucomutase and glucose-6-phosphate dehydrogenase; and measuring the amount of production of thus produced NAD(P)H to determine inorganic phosphorus.

(2) A process for measuring inorganic phosphorus characterized in: allowing a reaction between sucrose and inorganic phosphorus in the presence of sucrose phosphorylase; producing NAD(P)H from NAD(P) through combining the produced glucose-1-phosphate with phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; and measuring the amount of production of thus produced NAD(P)H to determine inorganic phosphorus.

3. Detailed Description of the Invention

(Field of Industrial Application)

The present invention relates to a process for measuring inorganic phosphorus which can obviate an influence of bilirubin, ascorbic acid, hemoglobin and the like, and which can be readily applied to an

automatic analyzer.

(Prior Art)

Conventionally, as a commonly employed process for measuring inorganic phosphate (Pi), a phosphomolybdic acid method has been known in which phosphomolybdic acid is reduced with inorganic phosphate (Pi) to measure as molybdenum blue. This process is not necessarily satisfactory because it is strongly affected by a reductive substance, and often causes the damage on the equipment because strong acid such as sulfuric acid is used.

In stead of such a method, some enzymatic measurement processes have been developed. Specifically, a process as described below has been known in which, for example, phosphorylase a is used.

$$\text{glycogen} + \text{Pi} \xrightarrow{\text{phosphorylase a}} \text{G-1-phosphate}$$

$$\text{G-1-phosphate} \xrightarrow{\text{phosphoglucomutase}} \text{G-6-phosphate}$$

$$\text{G-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6 phosphogluconate} + \text{NADP}$$

According to this process, molecular weight of glycogen is not constant, thereby involving drawbacks of difficulty in control.

Furthermore, a process in which a reaction of glycerin aldehyde-3-phosphate dehydrogenase is utilized has been also known. However, this process

has not been used so often.

As a process which is often used recently, there exists a process in which inosine and purine nucleoside phosphorylase are added to allow phosphorolysis resulting in the production of hypoxanthine, which is oxidized by xanthine oxidase to produce $H_2O_2$ followed by conjugation thereof in a peroxidase coloring system for permitting colorimetry. Although this process is extremely simple, influences of reductive substances such as bilirubin, ascorbic acid, hemoglobin and the like may be exerted, and is also disadvantageous in respect of being affected by chyle.

Moreover, most recently, a process in which inorganic phosphorus (Pi) is measured by quantitative determination of chlorine or ribose-1-phosphate generated through allowing a reaction of inorganic phosphorus (Pi) in the presence of nucleoside and nucleoside-phosphorylase is disclosed in the gazette of JP-A No. 60-160898.

(Problems to be Solved by the Invention)

The present inventors elaborately investigated in order to improve the conventional methods as described above, and consequently found a process for measuring inorganic phosphorus which can obviate an influence of bilirubin, ascorbic acid, hemoglobin and the like, and which can be readily applied to an automatic analyzer.

(Means for Solving the Problem)

First aspect of the present invention is a process for measuring inorganic phosphorus characterized in: allowing a reaction between sucrose and inorganic phosphorus in the presence of sucrose phosphorylase; producing NAD(P)H from NAD(P) through combining the produced glucose-1-phosphate with phosphoglucomutase and glucose-6-phosphate dehydrogenase; and measuring the amount of production of thus produced NAD(P)H to determine inorganic phosphorus.

Second aspect of the invention is a process for measuring inorganic phosphorus characterized in: allowing a reaction between sucrose and inorganic phosphorus in the presence of sucrose phosphorylase; producing NAD(P)H from NAD(P) through combining the produced glucose-1-phosphate with phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; and measuring the amount of production of thus produced NAD(P)H to determine inorganic phosphorus.

According to the present process, inorganic phosphorus (Pi) is quantitatively determined by measuring NAD(P)H which is finally generated by a reaction of inorganic phosphorus using sucrose and sucrose phosphorylase.

Next, the present process is explained by way of reaction formulae.

$$\text{Sucrose + Pi} \xrightarrow{\text{Sucrose phosphorylase}} \text{fructose + }\alpha\text{-D-}$$

glucose-1-phosphate

Further, α-phosphoglucomutase and glucose-6-phosphate dehydrogenase are combined as follows.

$$\alpha\text{-D-glucose-1-phosphate} + \text{D-glucose-1,6-diphosphate} \xrightarrow{\alpha\text{-phosphoglucomutase}} \text{D-glucose-6-phosphate} + \text{D-glucose-1,6-diphosphate}$$

$$\text{D-glucose-6-phosphate} \xrightarrow[\text{NAD(P)} \quad \curvearrowright \quad \text{NAD(P)H}]{\text{Glucose-6-phosphate dehydrogenase}} \text{6-phospho-D-gluconate}$$

By measuring the varying amount of NAD(P)H generated from NAD(P), inorganic phosphorus (Pi) can be quantitatively determined. Upon practice of the present process, the initial velocity method is preferred in order to obviate the influence of bilirubin, hemoglobin and the like in serum or urine, however, the terminal method is also permitted, of course, in which consumption of inorganic phosphorus (Pi) is rendered. Herein, NAD refers to nicotinamide adenine dinucleotide, while NADP refers to nicotinamide adenine dinucleotide phosphate.

Meanwhile, normal level of inorganic phosphorus in serum is low, i.e., 3 to 4.5 mg/dl. Therefore, a process with high sensitivity has been desired. Although the first process of the present invention as

described above can be sufficiently employed, further combination with 6-phosphogluconate dehydrogenase can lead to the generation of 2 mol of NAD(P)H per 1 mol of inorganic phosphorus (Pi), thereby resulting in sensitivity 2 times higher than the first process described above. This second process of the present invention is explained below by way of reaction formulae.

6-phospho-D-gluconate ⟶

6-phosphogluconate dehydrogenase

NAD(P) NAD(P)H

→ D-ribulose-5-phosphate + $CO_2$

6-phospho-D-gluconate ⟶

6-phosphogluconate dehydrogenase

NAD(P) NAD(P)H

→ 6-phospho-2-keto-D-gluconate

According to this second process of the present invention, it is advantageous in that amount of the sample can be diminished in order to avoid the influence of coloring substances such as bilirubin, hemoglobin and the like, and endogenous substances such as glucose and the like.

As a matter of course, buffer without contamination of inorganic phosphorus should be used, and examples of the buffer which may be used include

Tris, Tris-maleate, Tris-acetate, as well as PIPES [Piperazine-N,N′-bis(2-ethanesulfonic acid)], MES [2-(N-Morpholino)ethanesulfonic acid, monohydrate], BES [N,N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid], HEPES (N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid), TES [N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] and the like, which are referred to as good buffer. However, the buffer which may be used is not limited thereto, but any other buffer can be used, of course, as long as it does not include inorganic phosphorus and can maintain the buffering capability. Such buffer may be used at the concentration of 20 mM to 200 mM, and with the pH of 6.5 to 8.0.

Sucrose may be used at the concentration of preferably in the range of from 10 mM to 500 mM, and more preferably, at 80 mM to 200 mM.

Glucose-1,6-diphosphate is required for the activation of α-phosphoglucomutase, and is used preferably at the concentration of 0.01 to 0.2 mM. Depending on the quality of α-phosphoglucomutase, there may be instances in which activity is expressed without freshly adding glucose-1,6-diphosphate.

There exist glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase which are either dependent on NAD, or dependent on NADP, owing to the origin from which it is derived. Therefore, the enzyme should be selected whether the measurement is carried out in the NAD system or in the NADP system.

It is preferred that glucose-6-phosphate

dehydrogenase is used at the concentration of 1 to 10 U/ml, 6-phosphogluconate dehydrogenase at 0.1 to 1 U/ml, sucrose phosphorylase at 0.1 to 1 U/ml, α-phosphoglucomutase at 1 to 10 mM, and $Mg^{2+}$ at 1 to 10 mM, respectively. However, the range of concentration other than the aforementioned range can be also applied, as the condition may be.

Although the above explanation presents the ultraviolet ray absorption method for measuring NAD(P)H, measurement in the visible region is also possible. Specifically, measurement can be executed by coloring of NAD(P)H using a tetrazolium salt such as nitroblue tetrazolium (NTB), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (INT) or the like, in combination with an electron transfer system such as 1-methoxy-5-methylphenazinium sulfate (1-methoxy PMS), diaphorase or the like. This procedure is useful when a large scale treatment is carried out in accordance with the present process.

(Example)

The present process is more specifically explained below by way of Examples.

Example 1

This Example is directed to the first process of the present invention.

Composition of Reagents

| | |
|---|---|
| PIPES | 50 mM, pH 7.0 |
| sucrose | 200 mM |
| α-phosphoglucomutase | 2 U/ml |
| glucose-6-phosphate dehydrogenase | 5 U/ml |

| | |
|---|---|
| $MgCl_2$ | 3 mM |
| NAD | 3 mM |
| G-1,6-diphosphate | 0.05 mM |
| sucrose phosphorylase | 0.4 U/ml |

Reagents in the above composition in the amount of 2.5 ml were previously heated, and the sample in an amount of 50 µl was added thereto. Thereafter, ΔE/min was measured at 340 nm, and the linearity was determined. Linearity that passes the zero point was shown in the range of the inorganic phosphorus (Pi) of 50 mg/dl or less.

Example 2

This Example is directed to the second process of the present invention.

Composition of Reagents

| | |
|---|---|
| PIPES | 50 mM, pH 7.0 |
| sucrose | 200 mM |
| α-phosphoglucomutase | 2 U/ml |
| glucose-6-phosphate dehydrogenase | 5 U/ml |
| $MgCl_2$ | 3 mM |
| NAD | 3 mM |
| G-1,6-diphosphate | 0.05 mM |
| sucrose phosphorylase | 0.4 U/ml |
| 6-phosphogluconate dehydrogenase | 0.2 U/ml |

Reagents in the above composition in the amount of 2.5 ml were previously heated, and the sample in an amount of 20 µl was added thereto. Thereafter, ΔE/min was measured at 340 nm,

Determination of linearity

Satisfactory linearity that passes the zero point was shown in the range of the inorganic phosphorus (Pi) of 100 mg/dl or less.

Measurement of sample

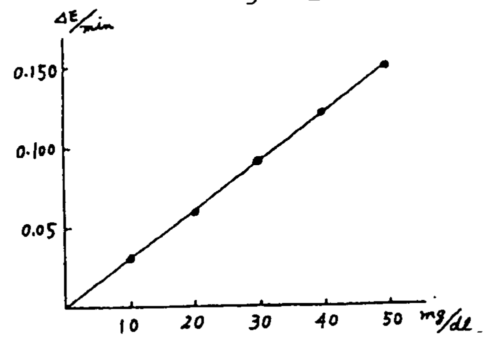
Comparison with the measurement value according to the conventional method (Fiske-Subbarow method) was conducted. The results are presented in Table 1.

| Sample | Conventional method | Example 2 |
|---|---|---|
| 1 | 7.5 mg/dl | 7.5 mg/dl |
| 2 | 2.1 | 1.9 |
| 3 | 20.0 | 22.1 |
| 4 | 5.3 | 5.1 |
| 5 | 3.5 | 3.4 |
| 6 | 6.1 | 6.3 |
| 7 | 10.3 | 10.2 |
| 8 | 13.6 | 13.3 |
| 9 | 15.1 | 15.2 |
| 10 | 4.1 | 3.9 |

4. Brief Description of Drawings

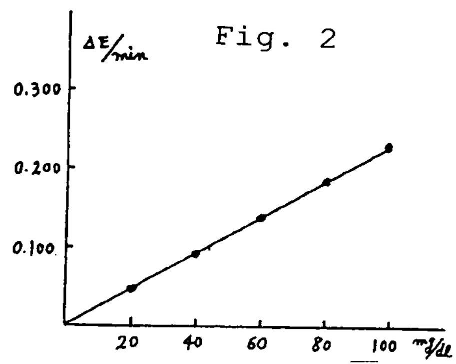
Fig. 1 is a drawing showing the linearity in Example 1 of the present invention; and Fig. 2 is a drawing showing the linearity in Example 2 of the present invention.

Fig. 1




amount of inorganic phosphorus

Fig. 2




amount of inorganic phosphorus